CYTOCHROME C OXIDASE ENZYME COMPLEX

FIELD OF THE INVENTION

[0001] The present invention relates to the production of a cytochrome c oxidase complex having cytochrome c oxidase activity. More particularly, present invention relates to recombinant production of 2-keto-L-gulonic acid and biologically useful materials thereof.

BACKGROUND OF THE INVENTION

[0002] Cytochrome c oxidase (cytochrome aa3; EC 1.9.3.1) is a terminal oxidase enzyme in the aerobic respiratory electron transport system of mitochondria and many bacteria. The enzyme is a cytoplasmic membrane spanning complex that catalyzes the final step in electron excretion involving the re-oxidation of ferrocytochrome c (electron donor) at the periplasmic surface and the reduction of molecular oxygen (electron acceptor) to water at the cytoplasmic surface. The reaction is coupled to the extrusion of protons across the membrane. This coupling is indispensable for the conservation of biological energy derived from substrate oxidation.

[0003] Various types of cytochrome complex, e.g. aa3, a1, caa3, o, bo, co, and bd-types, have been identified as functional terminal oxidases. The purification and characterization of some terminal oxidases has been reported. Matsushita et al. reported that *Acetobacter aceti* IFO 3283 contains two terminal oxidases, cytochrome a1 and o. (Proc. Natl. Acad. Sci., USA, 87: 9863, 1990; J. Bacteriol. 174: 122, 1992). (Id.) Matsushita et al. purified and characterized the cytochrome a1. Matsushita et al. also reported the purification of cytochrome o from *Gluconobacter* (Biochem. Biophys. Acta, 894: 304, 1987). Tayama et al. disclosed the terminal oxidase (cytochrome a1) genes of *A. aceti* (JP 93-317054) and they also purified the oxidase enzyme consisting of four subunits of 72, 34, 21, and 13 kDa and also containing heme

a and heme b. The oxidases in *Acetobacter* and *Gluconobacter* belong to quinol oxidase family of oxidases. Cytochrome aa3 (cytochrome c oxidase) has been purified from bovine heart, yeast, and many bacteria including *Paracoccous denitrificans* (Solioz et al., J. Biol. Chem., 257: 1579-1582, 1982) and *Rhodobacter sphaeroides* (Hosler et al., J. Biol. Chem., 267: 24264-24272, 1992).

[0004] Mammalian (mitochondrial) cytochrome c oxidase (aa3-type) complex contains 13 different subunits; the three core subunits I, II and III (CO I, II and III) are encoded by mitochondria DNA, while the remaining 10 subunits originate from the nucleus. Bacterial aa3-type cytochrome c oxidase also contains three core subunits that are homologous to the mitochondrial core subunits. However, it is reported that CO III was easily lost during purification, resulting in preparations composed of CO I and CO II only (Ludwig et al., Proc. Natl. Acad. Sci. USA, 77: 196-200, 1980). The cytochrome c oxidase complex consisting of the two-subunit (CO I and II) showed redox activity along with the generation of an electrochemical proton gradient. In the case of *P. denitrificans* (Haltia et al., The EMBO Journal, 10: 2015-2021, 1991) and *R. sphaeroides* (Cao et al., Gene, 101: 133-137, 1991), both two-subunit-type (CO I / II) and three-subunit-type (CO I / II / III) complexes were isolated by different purification methods. Genetically, genes for CO II and III are located in an operon, while the gene for CO I is independently located (Raitio et al., The EMBO Journal, 9: 2825-2833, 1987; Shapleigh et al., Proc. Natl. Acad. Sci. USA, 89: 4786-4790, 1992).

[0005] Terminal oxidases, as described above play an important role in cellular growth under aerobic conditions by accomplishing the reduction of the molecular oxygen. In oxidative fermentation, the respiratory chain, including the terminal oxidase, function by completing oxidation of a substrate to produce an oxidized product. In this context, it is very important to improve the efficiency of the respiratory chain in order to achieve efficient oxidative fermentation.

[0006] *G. oxydans* DSM 4025 produces 2-keto-L-gulonic acid (hereinafter: 2KGA), an important intermediate in the process of L-ascorbic acid production from L-sorbose via L-sorbosone (T. Hoshino et al., EP 0 366 922 A). The oxidation of the substrate, L-sorbose, to 2KGA was thought to be accomplished by the respiratory electron transport chain. The terminal oxidase that catalyzes the final electron excretion step via oxygen, might be one of the kinetic rate-limiting steps in the 2KGA production system as well as in the production of other redox components. The primary dehydrogenase responsible for 2KGA formation from L-sorbose was isolated (T. Hoshino et al., EP 606621 A) and the genes were cloned and

sequenced. Four isozymes of the primary dehydrogenase were found (T. Hoshino et al., EP 832974 A). Their direct electron acceptor, cytochrome c551, was also purified and its gene cloned (T. Hoshino et al., EP 0869175 A). However, the terminal oxidase was not isolated and its genes were not cloned.

SUMMARY OF THE INVENTION

[0007] The present invention is aimed at providing the materials for improving the quantity and quality of cytochrome c oxidase, and at improving oxidative fermentation completed by the cytochrome c oxidase by making novel cytochrome c oxidase genes available. The microorganism deposited as *Gluconobacter oxydans* under the accession No. DSM 4025 is the preferred source for providing the novel cytochrome c oxidase and the respective genetic materials of the present invention.

[0008] The present invention provides a novel cytochrome c oxidase enzyme complex that is isolated from a natural source or is prepared with the aid of genetic engineering. Such an enzyme complex having cytochrome c oxidase activity is obtainable or obtained from biological or genetic material originated from the microorganism identified as *G. oxydans* DSM 4025 or biologically and/or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of *G. oxydans* DSM 4025. Thus, the present invention provides a novel cytochrome c oxidase complex that is useful as an essential component mediating electron transfer in the respiratory chain.

[0009] The cytochrome c oxidase complex exemplified herein display the following physicochemical properties: (i) the presence of at least two core subunits of I (COI) and II (COII), wherein the apparent molecular mass of COI is about 43 +/- 10 kDa by SDS-PAGE analysis, and apparent molecular mass of COII is about 36 +/- 10 kDa by SDS-PAGE analysis; and (ii) the absorption spectrum showing aa3-type cytochrome c oxidase displays a 605 +/- 1 nm peak in reduced minus oxidized difference spectrum. Such a cytochrome c oxidase complex can be provided as a substantially homogeneous isolate derived from the culture of a microorganism identified as *G. oxydans* DSM 4025 or the biologically and/or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of *G. oxydans* DSM 4025.

[0010] The novel cytochrome c oxidase complex of the present invention can be also provided in the form of a recombinant enzyme, which may include a recombinant polypeptide as a core subunit I (COI), wherein the recombinant polypeptide is selected from the group of polypeptides having an amino acid sequence identified by SEQ ID NO:2, and those having amino acid sequences having 85% or higher identity with the sequence and which provides the complex with cytochrome c oxidase activity. Further, the other core subunit II (COII) and III (COIII) may be recombinant polypeptide(s) selected from the group of those containing amino acid sequences identified by SEQ ID NOs: 4, 6 and/or 8 and those containing amino acid sequences having 85% or higher identity with any one of the SEQ ID Nos: 4, 6 and 8, and that provide the complex with cytochrome c oxidase activity.

[0011] Another aspect of the present invention are the respective core subunits, *i.e.* COI, COII and COIII, that are recombinant polypeptides useful as components of the novel cytochrome c oxidase complex of the present invention.

[0012] Exemplified herein as COI is a recombinant polypeptide which is a component of the cytochrome c oxidase complex, the polypeptide having an amino acid sequence identified by SEQ ID NO: 2, or an amino acid sequence having 85 % or higher identity with the SEQ ID NO:2 and that provides the complex with cytochrome c oxidase activity. The recombinant COI may be a polypeptide capable of providing the complex of the present invention with cytochrome c oxidase activity, and that is encoded by a recombinant DNA fragment containing a DNA sequence selected from the group of:

(a) the DNA sequence identified by SEQ ID NO: 1, and

(b) DNA sequences that encode polypeptides having an amino acid sequence identified by SEQ ID NO: 2 or amino acid sequences having 85 % or higher identity with SEQ ID NO:2.

[0013] Also exemplified herein as COII is a recombinant polypeptide which is a component of the cytochrome c oxidase complex of the present invention, the polypeptide having an amino acid sequence identified by SEQ ID NO: 4, or an amino acid sequence having 85 % or higher identity with the amino acid sequence and that is capable of providing the complex with cytochrome c oxidase activity. The recombinant COII may be a polypeptide capable of providing the complex of the present invention with cytochrome c oxidase activity, and that is encoded by a recombinant DNA fragment containing a DNA sequence selected from the group of:

(a) the DNA sequence identified by SEQ ID NO: 3, and

(b) the DNA sequences that encode polypeptides having an amino acid sequence identified by SEQ ID NO: 4 or an amino acid sequence having 85% or higher identity with SEQ ID NO:4.

[0014] Moreover, exemplified herein as COIII is a recombinant polypeptide that is a component of the cytochrome c oxidase complex of the present invention. Such recombinant polypeptide contains either, or both of the amino acid sequences identified by SEQ ID NOs: 6 and 8, respectively or amino acid sequences having 85% or higher identity with SEQ ID NOs: 6 and 8 and that provide the complex with cytochrome c oxidase activity. The recombinant COIII may be a recombinant polypeptide capable of providing the complex of the present invention with cytochrome c oxidase activity, that is encoded by a recombinant DNA fragment containing one or more DNA sequence(s) selected from the group of:

(a) the DNA sequence identified by SEQ ID NO: 5,

(b) the DNA sequence identified by SEQ ID NO: 7,

(c) the DNA sequences that encode polypeptides having an amino acid sequence identified by SEQ ID NO: 6 or amino acid sequences having 85% or higher identity with the SEQ ID NO:6, and

(d) the DNA sequences that encode polypeptides having an amino acid sequence identified by SEQ ID NO: 8 or amino acid sequences having 85% or higher identity with SEQ ID NO:8.

[0015] Further aspects of the present invention are recombinant DNA fragments useful for preparing the respective core subunits, i.e. COI, COII and COIII by genetic engineering. Such recombinant polypeptides are useful as components of the novel cytochrome c oxidase complex of the present invention. As explained above, these polypeptides should be capable of providing the cytochrome c oxidase complex of the present invention with cytochrome c oxidase activity

[0016] Exemplified herein as a recombinant DNA fragment for COI is a DNA fragment which encodes a polypeptide involved in the cytochrome c oxidase complex, and that includes a DNA sequence selected from the group of:

(a) the DNA sequence identified by SEQ ID NO: 1, and

(b) DNA sequences that encode polypeptides having an amino acid sequence identified by SEQ ID NO: 2 or amino acid sequences having 85% or higher identity with SEQ ID NO:2.

[0017] Also exemplified herein as a recombinant DNA fragment for COII is a DNA fragment that encodes a polypeptide involved in the cytochrome c oxidase complex and that contains a DNA sequence selected from the group of:

(a) the DNA sequence identified by SEQ ID NO: 3, and

(b) DNA sequences that encode polypeptides having an amino acid sequence identified by SEQ ID NO: 4 or amino acid sequences having 85% or higher identity with SEQ ID NO:4.

[0018] Also exemplified herein as a recombinant DNA fragment for COIII is a DNA fragment that encodes a polypeptide involved in the cytochrome c oxidase complex and that contains one or more DNA sequence(s) selected from the group of:

(a) the DNA sequence identified by SEQ ID NO: 5,

(b) the DNA sequence identified by SEQ ID NO: 7,

(c) DNA sequences that encode polypeptides having an amino acid sequence identified by SEQ ID NO: 6 or amino acid sequences having 85% or higher identity with SEQ ID NO:6, and

(d) DNA sequences that encode polypeptides having an amino acid sequence identified by SEQ ID NO: 8 or amino acid sequences having 85% or higher identity with SEQ ID NO:8.

[0019] Another aspect of this invention is an expression vector containing one or more of the above mentioned recombinant DNA fragments, the vector being suitable for expression in an organism, including both prokaryotic and/or eukaryotic host cells.

[0020] Further, another aspect of the present invention is a recombinant organism into which has been introduced the expression vector mentioned above. Such a recombinant organism is useful for the genetic preparation of the recombinant cytochrome c oxidase

complex and also applicable to a process for producing 2KGA from L-sorbose or D-sorbitol in an appropriate culture medium. Host cells for the recombinant organism of the present invention may be of eukaryotic origin, preferably a mammalian or plant cell, or may be of prokaryotic origin. These host cells may in particular be obtained from bacteria, preferably *G. oxydans* DSM 4025 and biologically and/or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of *Gluconobacter oxydans* DSM 4025.

[0021] This invention is also directed to a process for producing cytochrome c oxidase, which includes cultivating the recombinant organism of this invention, as mentioned above, particularly the recombinant organism containing a preferred DNA sequence exemplified herein, in an appropriate culture medium and recovering the cytochrome c oxidase from the culture medium.

[0022] Further, this invention is also directed to a process for producing 2KGA from L-sorbose or D-sorbitol, which includes cultivating a recombinant organism of the present invention, as mentioned above, in an appropriate culture medium and recovering 2KGA from the culture.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] The following figures are included to further illustrate the present invention together with the detailed description given below.

[0024] Figure 1 shows absorption spectra pattern of aa3-type cytochrome c oxidase of *G. oxydans* DSM 4025. Spectra were recorded at room temperature at a protein concentration of 0.08 mg/ml in 25 mM Na-HEPES (pH 7.5) containing 0.5% sucrose monolaurate and 5% glycerol. Figure 1A shows the spectrum of the oxidized form. Figure 1B shows the spectrum of the reduced form. Figure 1C shows the reduced minus oxidized difference spectrum.

[0025] Figure 2 shows SDS-PAGE analysis of the purified cytochrome c oxidase aa3 of *G. oxydans* DSM 4025. The purified enzyme (at a protein concentration of 0.5 mg/ml) was denatured by incubation with 2% SDS, 50 mM dithioerythritol, 62.5 mM Tris-HCl (pH 6.8) and 10% glycerol at 37°C for 5 hours. Electrophoresis was carried out at 12.5% acrylamide concentration according to the method of Laemmli (Nature, 227: 680-685, 1970) with the buffer consisting of 25 mM Tris, 0.192 M glycine, and 0.1% SDS. Lane A and B contain 6

microgram and 3 microgram of the purified enzyme, respectively. Lane C contains low range prestained SDS-PAGE standards (Bio-Rad Laboratories, CA U.S.A.).

[0026] Figure 3 shows an alignment of the partial amino acid sequences of CO I from *G. oxydans* DSM 4025 with ones from other organisms.

[0027] Figure 4 shows an alignment of the partial amino acid sequences of CO II from *G. oxydans* DSM 4025 with ones from other organisms.

[0028] Figure 5 shows an alignment of the partial amino acid sequences of CO III from *G. oxydans* DSM 4025 with those from other organisms.

[0029] Figure 6 shows primers for PCR amplification of the partial CO I, II and III genes of the cytochrome c oxidase complex from *G. oxydans* DSM 4025.

[0030] Figure 7 shows the physical maps of the 8.0 kb PstI and 9.3 kb EcoRI fragments containing the "CO I" and "CO II and III" genes, respectively.

[0031] Figure 8 shows an alignment of the complete amino acid sequence of the CO I subunit from *G. oxydans* DSM 4025 with those from other organisms.

[0032] Figure 9 shows a genetic map of the pVKcoxes construct used for expressing the genes of cytochrome c oxidase complex of *G. oxydans* DSM 4025.

DETAILED DESCRIPTION OF THE DRAWINGS

[0033] The novel cytochrome c oxidase complex of the present invention belongs to a family of proteins, and corresponding genes, that function as a terminal oxidases. More particularly, the novel cytochrome c oxidase of the present invention is useful as a terminal oxidase that oxidizes cytochrome c, an electron acceptor for dehydrogenases, such as alcohol and aldehyde dehydrogenase (AADH), and thus, is useful as an essential component mediating electron transfer in the respiratory chain. The cytochrome c oxidase complex of the present invention may be isolated from a natural source or prepared with the aid of genetic engineering. Such an enzyme complex having cytochrome c oxidase activity is obtainable from biological material originated from a microorganism identified as *G. oxydans* DSM 4025 or biologically and/or taxonomically homogeneous cultures of a microorganism having the identifying

characteristics of *G. oxydans* DSM 4025. The cytochrome c oxidase complex of the present invention shows the following physico-chemical characteristics: the complex shows the absorption spectra of aa3-type cytochrome c oxidase in reduced minus oxidized difference spectrum (a peak at 605 +/- 1 nm) and, two polypeptides involved in the cytochrome c oxidase complex have apparent molecular masses of about 43 +/- 10 kDa and 36 +/- 10 kDa on SDS-PAGE.

[0034] As used herein, the phrase "a biologically and/or taxonomically homogeneous culture of a microorganism having the identifying characteristics of *G. oxydans* DSM 4025" means a microorganism that has at least 12 out of 14 of the following characteristics of *G. oxydans* DSM 4025:

(a) produces 2-KGA from L-sorbose,

(b) oxidizes ethanol to acetic acid,

(c) oxidizes D-glucose to D-gluconic acid and 2-keto-D-gluconic acid,

(d) exhibits ketogenesis of polyalcohols,

(e) exhibits pellicle and ring growth in mannitol broth (24 hour cultivation) at pH 4 and 5, and pellicle growth in glucose broth at pH.45,

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(f) does not substantially oxidize glycerol to dihydrooxyacetone,

(g) produces 2-keto-D-glucaric acid from sorbitol and glucaric acid but not from glucose, fructose, gluconic acid, mannitol or 2-keto-D-gluconic acid,

(h) is polymorphic, with no apparent flagella,

(i) produces brown pigment from fructose,

(j) exhibits good growth when co-cultured in the presence of B. megaterium or a cell extract thereof,

(k) is streptomycin sensitive,

(l) is rod-shaped with rounded ends,

(m)has an average cell diameter of about 0.3-0.6 micrometers,

(n) has an average cell length of about 1-1.5 micrometers; and

which microorganism produces 2-KGA from L-sorbose on the level of at least 0.01 g/L of 2-KGA in the culture medium as measured by HPLC. In addition to this, the phrase "a biologically and/or taxonomically homogeneous culture of a microorganism having the identifying characteristics of *G. oxydans* DSM 4025" should be understood to encompass a microorganism comprising a polynucleotide sequence which hybridizes under high stringency conditions to a polynucleotide sequence which encodes a polypeptide selected from the group consisting of SEQ ID NO:2, 4, 6, and 8, as it is obvious for the person skilled in the art that such a microorganism can be identified based on homology of the amino acid sequences.

[0035] The novel recombinant enzyme complex of the present invention can be prepared using genetic material, i.e. recombinant DNA fragments originated from a microorganism identified as *G. oxydans* DSM 4025 or a biologically and/or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of *G. oxydans* DSM 4025. Such a novel cytochrome c oxidase complex may contain at least one recombinant polypeptide as one of the core subunits. The recombinant polypeptide as the core subunit I of the complex may be selected from the group of polypeptides having an amino acid sequence identified by SEQ ID NO: 2 and those amino acid sequences having 85% or higher identity with SEQ ID NO:2 and being capable of providing the complex with cytochrome c oxidase activity. Furthermore, either or both of the other core subunits, II (COII) and III (COIII), may be recombinant polypeptide(s). COII may be selected from the group consisting of recombinant polypeptides containing a partial amino acid sequence identified by SEQ ID NO: 4, and those containing a partial amino acid sequence having 85% or higher identity with SEQ ID NO:4, as long as such recombinant polypeptides are capable of providing the complex with cytochrome c oxidase activity. COIII may be selected from the group of recombinant polypeptides containing partial amino acid sequences identified by SEQ ID NOs: 6 and 8 and those containing partial amino acid sequences having 85% or higher identity with SEQ ID NOs:6 and 8, respectively, as long as such recombinant polypeptides are capable of providing the complex with cytochrome c oxidase activity.

[0036] The term "identity" preferably has the meaning that the amino acids occurring at the respective positions are not only similar with regard to their properties, but are in fact identical. In a preferred embodiment the alignment of the amino acid sequences is performed, for example, using the GCG alignment program in Best Fit.

[0037] As used herein, % homology data are generated using the "Search Homology" program of Genetyx-SV/RC version 3.2.0 (Genetyx Software Development Co. Ltd., Tokyo, Japan).

[0038] The present invention is also directed to the polypeptides involved in the cytochrome c oxidase complex. The polypeptides involved in the cytochrome c oxidase complex and the amino acid sequences described in SEQ ID NOs: 2, 4, 6 and 8 displayed homologies of 50-82%, at most, with the polypeptides or the corresponding partial amino acid sequences, involved in other cytochrome oxidases. For example, the CO I polypeptide of the present invention (SEQ ID NO: 2) displayed 77%, 81% and 79% homology with CO I alpha (accession No. P08305), CO I beta (accession No. P98002) from *P. denitrificans* and CO I from *R. sphaeroides* (accession No. P33517), respectively. The partial CO II polypeptide of the present invention (SEQ ID NO: 4) displayed 73% and 68% homology with the CO II polypeptides from *P. denitrificans* and *R. sphaeroides*, respectively. One of the partial CO III polypeptides of the present invention (SEQ ID NO: 6) displayed 54% homology with the CO III polypeptide from *P. denitrificans* and another polypeptide (SEQ ID NO: 8) displayed 71% and 63% homology with the CO III polypeptides from *P. denitrificans* and *R. sphaeroides*, respectively. These homology searches can be done by a computer program such as "Search Homology" of Genetyx-SV/RC version 3.2.0 (Genetyx Software Development Co. Ltd., Tokyo Japan).

[0039] Thus the respective core subunits, i.e. COI, COII and COIII may be provided as recombinant polypeptides which are useful as components of the novel cytochrome c oxidase complex of the present invention.

[0040] The subunit COI of the complex may be a recombinant polypeptide which is a component of the cytochrome c oxidase complex of the present invention, the polypeptide having an amino acid sequence identified by SEQ ID NO: 2 or an amino acid sequence having 85% or higher identity with SEQ ID NO:2, and that is capable of providing the complex with cytochrome c oxidase activity, as described above. The recombinant COI may also be a polypeptide that provides the complex of the present invention with cytochrome c oxidase activity, and that is encoded by a recombinant DNA fragment comprising a DNA sequence selected from the group consisting of:

(a) the DNA sequence identified by SEQ ID NO: 1, and

(b) DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 2 or amino acid sequences having 85% or higher identity with SEQ ID NO:2.

[0041] Also, the subunit COII may be a recombinant polypeptide which is a component of the cytochrome c oxidase complex of the present invention, the polypeptide having an amino acid sequence identified by SEQ ID NO: 4, or an amino acid sequence having 85% or higher identity with SEQ ID NO:4, and that provides the complex with cytochrome c oxidase activity, as described above. A recombinant COII may also be a polypeptide that provides the complex of the present invention with cytochrome c oxidase activity, and that is encoded by a recombinant DNA fragment containing a DNA sequence selected from the group of:

(a) the DNA sequence identified by SEQ ID NO: 3, and

(b) DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 4 or amino acid sequences having 85% or higher identity with SEQ ID NO:4.

[0042] The subunit COIII may be a recombinant polypeptide which is a component of the cytochrome c oxidase complex of the present invention, the polypeptide having either or both of the amino acid sequences identified by SEQ ID NOs: 6 and 8, respectively, or amino acid sequences having 85% or higher identity with SEQ ID NOs: 6 and 8, respectively, as long as such recombinant polypeptides provide the complex with cytochrome c oxidase activity, as described above. A recombinant COIII may also be a recombinant polypeptide capable of providing the complex of the present invention with cytochrome c oxidase activity, that is encoded by a recombinant DNA fragment containing one or more DNA sequence(s) selected from the group of:

(a) the DNA sequence identified by SEQ ID NO: 5,

(b) the DNA sequence identified by SEQ ID NO: 7,

(c) DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 6 or amino acid sequences having 85% or higher identity with SEQ ID NO:6, and

(d) DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 8 or amino acid sequences having 85% or higher identity with SEQ ID NO:8.

[0043] The present invention also encompasses functional derivatives of the recombinant polypeptides described above. As used herein, "functional derivatives" are defined, on the basis of the amino acid sequence of the present invention, by addition, insertion, deletion and/or substitution of one or more amino acid residues of such sequences, such as from 1 to 20 exchanges, for example from 2 to 10, 3-5, or 2-3 exchanges, where the cytochrome c oxidase complex including such derivatives still have cytochrome c oxidase activity measured by an assay known in the art or specifically described herein. Such functional derivatives may be made either by chemical peptide synthesis or chemical modification of protein known in the art, or by recombinant means on the basis of the DNA sequences as disclosed herein, by methods known in the state of the art and disclosed, e.g. by Sambrook et al (supra) ("Molecular Cloning" second edition, Cold Spring Harbour Laboratory Press 1989, New York). Amino acid exchanges in proteins and peptides which do not generally alter the activity of such molecules are known in the state of the art and are described, for example, by H. Neurath and R.L. Hill in "The Proteins" (Academic Press, New York, 1979, see especially Figure 6, page 14). The most commonly occurring exchanges are: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly, as well as these in reverse.

[0044] The present invention is directed to recombinant DNA fragments that encode recombinant polypeptides involved in the cytochrome c oxidase complex that is one of the essential components mediating electron transfer in the respiratory chain.

[0045] The recombinant DNA fragments which are useful for preparing the respective core subunits, i.e. COI, COII and COIII, by genetic engineering are provided. Such recombinant polypeptides are useful as components of the novel cytochrome c oxidase complex of the present invention.

[0046] A recombinant DNA fragment for COI may be a DNA fragment that encodes a polypeptide involved in the cytochrome c oxidase complex and that includes a DNA sequence selected from the group of:

(a) the DNA sequence identified by SEQ ID NO: 1, and

(b) DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 2, or amino acid sequences having 85% or higher identity with SEQ ID NO:2.

[0047] A recombinant DNA fragment for COII may be a DNA fragment which encodes a polypeptide involved in the cytochrome c oxidase complex and that contains a DNA sequence selected from the group of:

(a) the DNA sequence identified by SEQ ID NO: 3, and

(b) DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 4, or amino acid sequences having 85% or higher identity with SEQ ID NO:4.

[0048] A recombinant DNA fragment for COIII may be a DNA fragment which encodes a polypeptide involved in cytochrome c oxidase complex and contains one or more DNA sequence(s) selected from the group of:

(a) the DNA sequence identified by SEQ ID NO: 5,

(b) the DNA sequence identified by SEQ ID NO: 7,

(c) DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 6, or amino acid sequences having 85% or higher identity with SEQ ID NO:6, and

(d) DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 8, or amino acid sequences having 85% or higher identity with SEQ ID NO:8.

[0049] The recombinant DNA fragment of this invention may also include a DNA sequence which is capable of hybridizing to SEQ ID NO: 1, 3, 5, or 7 under standard stringency conditions which are described in more detail below.

[0050] As used herein the phrase "standard conditions for hybridization" means conditions which are generally used by a person skilled in the art to detect specific hybridization signals and which are described, e. g. by Sambrook et al (supra), or preferably, so called stringent hybridization and non-stringent washing conditions, or more preferably, so called moderately

stringent conditions, or even more preferably, so called stringent hybridization and stringent washing conditions which a person skilled in the art is familiar with and which are described, e.g. in Sambrook et al (supra).

[0051] For example, any combination of the following hybridization and wash conditions may be used, as appropriate:

High Stringency Hybridization:

6X SSC

0.5% SDS

100 ug/ml denatured salmon sperm DNA

50% formamide

Incubate overnight with gentle rocking at 42EC overnight.

High Stringency Wash:

1 wash in 2X SSC, 0.5% SDS at Room Temperature for 15 minutes,

followed by another wash in 0.1X SSC, 0.5% SDS at Room Temperature for 15 minutes.

Low Stringency Hybridization:

6X SSC

0.5% SDS

100 ug/ml denatured salmon sperm DNA

50% formamide

Incubate overnight with gentle rocking at 37EC overnight.

Low Stringency Wash:

1 wash in 0.1X SSC, 0.5% SDS at Room Temperature for 15 minutes.

[0052] Moderately stringent conditions may be obtained by varying the temperature at which the hybridization reaction occurs and/or the wash conditions as set forth above.

[0053] The present invention also provides an expression vector containing one or more of the above mentioned recombinant DNA fragments. The vector is suitable for the expression in an organism, including both prokaryotic- or eukaryotic host cells. Such an expression vector is constructed by inserting one or more of the above mentioned recombinant DNA fragments into a suitable vector which may carry expression control elements, as is well known in the art. As used herein, expression control elements include enhancers and cis elements to which trans-acting factors bind to control gene expression.

[0054] Further, a recombinant organism of the present invention may be prepared by introducing an expression vector mentioned above to an appropriate host cell. Such a recombinant organism of the invention would be useful for the genetic preparation of the recombinant cytochrome c oxidase complex of the present invention and also applicable to a process for producing 2KGA from L-sorbose or D-sorbitol in an appropriate culture medium. Host cells for the recombinant organism of the present invention may be of eukaryotic origin, preferably a mammalian or plant cell, or may be of prokaryotic origin. These host cells may in particular be obtained from bacteria, preferably *G. oxydans* DSM No. 4025 and biologically and/or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of *G. oxydans* DSM 4025. Host cells may also be selected from the group consisting of bacteria, such as *Escherichia coli, Pseudomonas putida, Acetobacter xylinum, Acetobacter pasteurianus, Acetobacter aceti, Acetobacter hansenii*, and *G. oxydans*.

[0055] In addition, the present invention also provides a process for producing cytochrome c oxidase. This process includes cultivating a recombinant host cell, as defined above, in an appropriate culture medium and recovering the cytochrome c oxidase from the culture.

[0056] The cytochrome c oxidase complex of the present invention is also used for improving 2KGA production from L-sorbose or D-sorbitol and also in the production of aldehydes, carboxylic acids, and ketones from corresponding substrates in the presence of alcohol and aldehyde dehydrogenase *in vivo* and *in vitro*.

[0057] The compound 2KGA is an important intermediate for the production of L-ascorbic. The production of 2KGA from L-sorbose, or from D-sorbitol by fermentation is known (T.

Hoshino et al., EP 88116156 A). Gluconobacter strains are known to produce 2KGA via the reaction catalyzed by sorbose and sorbosone dehydrogenases as disclosed in Agric. Biol. Chem., 54(5), 1211-1218, 1990 (T. Hoshino et al.) and in EP 606621 A (T. Hoshino et al.). The genes of primary dehydrogenases responsible for 2KGA formation from L-sorbose or D-sorbitol have been isolated (T. Hoshino et al., EP 832974 A). Furthermore, the cytochrome c that functions as an electron acceptor of the primary dehydrogenases as well as its gene have also been isolated (T. Hoshino et al., EP 869175 A). These dehydrogenases and cytochrome c have been used to produce 2KGA *in vitro*. The genes have been used to construct recombinant organisms producing 2KGA from L-sorbose and D-sorbitol; e.g. Pseudomonas putida carrying the genes of alcohol/aldehyde dehydrogenase (AADH) together with cytochrome c can produce 2KGA from L-sorbose.

[0058] Therefore, the present invention includes the use of the cytochrome c oxidase set forth above for the production of 2KGA.

[0059] The terminal oxidase activity of the present cytochrome c oxidase complex was spectrophotometrically measured using TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride) as an artificial substrate (electron donor). The reaction mixture consists of 2.5 mM TMPD, 0.05% Tween-20 and 0.1 M sodium 3[N-morpholino]propanesulfonic acid (Na-MOPS) (pH 6.5). The TMPD oxidase activity can be measured by increasing of absorption at 520 nm with the mole coefficient of TMPD taken as 6.1 /mM/cm. One unit of enzyme activity is defined as 1 micromole oxidation of TMPD per one minute at room temperature.

[0060] Spectrophotometric identification and quantification of a-type heme were carried out by detection of the characteristic positive peak around 605 nm by reduced minus oxidized difference spectrum. Reduction of each sample was carried out by the addition of a tiny amount of sodium dithionite and by oxidation with ammonium persulfate. The mole coefficient of the a-type heme peak (605 nm - 630 nm) was taken as 11.7 /mM/cm.

[0061] Before describing the present invention in more detail the physico-chemical properties of purified cytochrome c oxidase consisting of subunits, COI and COII, as obtainable from *G. oxydans* DSM 4025 are given below.

(1) Absorption spectrum

The absorption profile of the cytochrome c oxidase complex in reduced minus oxidized difference spectra is shown in Fig. 1.

(2) Molecular weight

SDS-PAGE analysis indicated apparent molecular masses of about 43 +/- 10 and 36 +/- 10 kDa for the cytochrome c oxidase CO I and CO II subunits, respectively, as shown in Fig. 2.

(3) Amino acid sequences of the CO I and CO II

[0062] The cytochrome c oxidase complex purified was dissociated into CO I and II subunits by a preparative-disc-SDS-PAGE (NA-1800, Nippon Eido Co,.). Both N-terminal alpha-amino residues were blocked by unidentified modification. Partially digested peptide fragments (15 - 45 kDa MW.) were then obtained by lysyl-endopeptidase treatment, isolated by band extraction from a 15% SDS-PAGE sheet, washed in a Centricon-10(Amicon) with 15% methanol and 0.1% SDS, and applied to the sequencer. "KDIGLLYLVAAGVVGF" (SEQ ID NO: 11) and "KASQFTHNTPLEIVWTIVPV" (SEQ ID NO: 14) sequences were obtained for CO I and COII, respectively.

[0063] The preferred strain used for isolating polypeptides and genes of cytochrome c oxidase of the present invention is the *G. oxydans* strain that deposited at the Deutsche Sammlung von Mikroorganismen in Göttingen (Germany) under DSM 4025 on March 17, 1987 under the stipulations of the Budapest Treaty. Moreover, a subculture of the strain has also been deposited in the Agency of Industrial Science and Technology, Fermentation Research Institute, Japan, under the stipulations of the Budapest Treaty under the deposit No.: *Gluconobacter oxydans* FERM BP-3812 (date of deposit: March 30, 1992). Furthermore, EP 278 447, which is hereby incorporated by reference as if recited in full herein, discloses the characteristics of this strain. Functional equivalents, subcultures, mutants and variants of said microorganism can also be used in the present invention. Biologically or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of the strain DSM 4025 can also be used as the source of the polypeptides and genes of the said cytochrome c oxidase.

[0064] The cytochrome c oxidase provided by the present invention may be prepared by cultivating an appropriate organism, disrupting the cells and isolating and purifying it from a cell free extract of disrupted cells, preferably from the soluble fraction of the organism.

[0065] The organisms may be cultured in an aqueous medium supplemented with appropriate nutrients under aerobic conditions. The cultivation may be conducted at a pH

between about 4.0 and 9.0, preferably between about 6.0 and 8.0. While the cultivation period varies depending upon pH, temperature and nutrient medium used, usually 2 to 6 days will bring about favorable results. A preferred temperature range for carrying out the cultivation is from about 13° to 36°C, preferably from about 18° to 33°C.

[0066] It is usually required that the culture medium contains such nutrients as assimilable carbon sources, digestible nitrogen sources and inorganic substances, vitamins, trace elements and the other growth promoting factors. As assimilable carbon sources, glycerol, D-glucose, D-mannitol, D-fructose, D-arabitol, L-sorbose, D-sorbitol and the like can be used.

[0067] Various organic or inorganic substances may also be used as nitrogen sources, such as yeast extract, meat extract, peptone, casein, corn steep liquor, urea, amino acids, nitrates, ammonium salts and the like. As inorganic substances, magnesium sulfate, potassium phosphate, ferrous and ferric chlorides, calcium carbonate and the like may be used.

[0068] Preferred, embodiments for the isolation and purification of cytochrome c oxidase from the organisms after their cultivation and for the cloning of the gene/DNA sequence are described.

(1) Cells are harvested from fermentation broth by centrifugation or filtration.

(2) The cells are suspended in buffer solution and disrupted by means of a homogenizer, sonicator or treatment with lysozyme and the like, to give a disrupted solution of cells.

(3) Cytochrome c oxidase is isolated and purified from a cell free extract of disrupted cells, preferably from the soluble fraction of the organisms by usual protein purification methods such as ammonium sulfate precipitation, dialysis, ion exchange chromatography, gel filtration chromatography, and affinity chromatography.

[0069] The cytochrome c oxidase as provided by the present invention is useful as a terminal oxidase oxidizing cytochrome c that functions as an electron acceptor from a dehydrogenase enzyme in the production of aldehydes, carboxylic acids and ketones from alcohols and aldehydes, especially for the production of 2KGA from L-sorbose or D-sorbitol via L-sorbosone.

[0070] Briefly, the cytochrome c oxidase genes, the DNA sequences, the recombinant expression vector and the recombinant organism, also referred to as the transformed host cell, utilized in the present invention can be obtained by the following steps:

(1) Isolating chromosomal DNA from the organisms that can provide the cytochrome c oxidase of the present invention and constructing a gene library of the chromosomal DNA in *Escherichia coli*.

(2) Cloning cytochrome c oxidase genes from chromosomal DNA by colony-, plaque- or Southern-hybridization, PCR (polymerase chain reaction) cloning, Western-blot analysis and the like.

(3) Determining the nucleotide sequences of the cytochrome c oxidase genes obtained as above by utilizing accepted methods to select recombinant DNA fragments containing the cytochrome c oxidase genes constructing an expression vector on which cytochrome c oxidase genes can be efficiently expressed.

(4) Constructing recombinant organisms carrying the cytochrome c oxidase genes by transformation, transduction, transconjugation and electroporation.

[0071] The materials and the techniques used in the above aspect of the present invention are exemplified, in detail, as follows:

[0072] Total chromosomal DNA may be purified by a procedure well known in the art. The genes encoding cytochrome c oxidase may be cloned in either plasmid or phage vectors from total chromosomal DNA by the following methods:

(i) by determining the partial amino acid sequences of the purified cytochrome c oxidase subunits by isolating the whole protein or peptide fragments, obtained by peptidase-treatment of the gel after SDS-polyacrylamide gel electrophoresis and applying them to a protein sequencer such as Applied Biosystems automatic gas-phase sequencer 470A (Perkin Elmer Corp., Norwalk, Conn., USA), synthesizing oligonucleotide probes with a DNA synthesizer such as Applied Biosystems automatic DNA sequencer 381A (Perkin Elmer), such oligonucleotide probes corresponding to the amino acid sequences obtained as above, isolating clones carrying the objective by utilizing the oligonucleotide probes to perform southern-, colony-, or plaque hybridization on a gene library of the strain carrying the objective genes; (ii) by selecting clones expressing cytochrome c oxidase subunits from the gene library by

immunological methods using antibodies against the subunits of cytochrome c oxidase; or (iii) by amplifying the DNAs from the total chromosomal DNA by the PCR method using pairs of oligonucleotides synthesized according to the amino acid sequences determined as above, and isolating clones carrying the whole genes of cytochrome c oxidase subunits from a gene library constructed in *E. coli* by Southern-, colony-, or plaque-hybridization using the PCR product obtained above as the probe. The above-mentioned antibodies that react against the subunits of cytochrome c oxidase may be prepared using the purified proteins of cytochrome c oxidase subunits, or their peptide fragments, by a method such as that described in Methods in Enzymology, vol. 73, p 46, 1981.

[0073] The nucleotide sequences of the cytochrome c oxidase genes may be determined by a well-known method, such as the dideoxy chain termination method using M13 phage (Sanger F. et al., Proc. Natl. Acad. Sci. USA, 74:5463-5467, 1977).

[0074] To express the genes of cytochrome c oxidase complex subunits, various promoters may be used; for example, the original promoter existing upstream of the genes for cytochrome c oxidase subunits, promoters of antibiotic resistance genes such as the kanamycin resistance gene of Tn5 (Berg, D. E., and C. M. Berg. 1983. Bio/Technology 1: 417-435), the ampicillin resistance gene of pBR322, the beta-galactosidase gene of *E. coli* (lac), the trp-, tac-, and trc-promoters, promoters of lambda phage and any promoters that are functional in a host consisting of organisms including bacteria such as *E. coli, P. putida, A. xylinum, A. pasteurianus, A. aceti, A. hansenii,* and *G. oxydans, especially G. oxydans* DSM 4025, mammalian cells and plant cells.

[0075] Other regulatory elements may be included, such as a Shine-Dalgarno (SD) sequence (for example, AGGAGG etc., including natural and synthetic sequences operable in the host cell) and a transcriptional terminator (inverted repeat structure including any natural and synthetic sequence operable in the host cell) that are operable in the host cell into which the coding sequence will be introduced and used with the above described promoter.

[0076] A wide variety of host/cloning vector combinations may be employed in cloning the double-stranded DNA. The cloning vector is generally a plasmid or phage which contains a replication origin, regulatory elements, a cloning site including a multi-cloning site, and selection markers such as antibiotic resistance genes including resistance genes for ampicillin, tetracycline, kanamycin, streptomycin, gentamicin, spectinomycin etc.

[0077] Preferred vectors for the expression of the object gene in *E. coli* are selected from any vectors usually used in *E. coli*, such as pBR322 or its derivatives, including pUC18 and pBluescript II, pACYC177 and pACYC184 (J. Bacteriol., 134:1141-1156, 1978) and their derivatives, and a vector derived from a broad host range plasmid such as RK2 or RSF1010. A preferred vector for the expression of the subject gene in *Gluconobacter* including *G. oxydans* DSM 4025 and *P. putida*, is selected from any vectors which can replicate in *Gluconobacter* and/or *P. putida*, as well as a in preferred cloning organism such as *E. coli*. The preferred vector is a broad-host-range vector, such as a cosmid vector like pVK102 and its derivatives, and RSF1010 and its derivatives, and a vector containing a replication origin functional in *Gluconobacter* and another origin functional in *E. coli*. The copy number and stability of the vector should be carefully considered for stable and efficient expression of the cloned gene and also for efficient cultivation of the host cell carrying the cloned gene. DNA sequences containing transposable elements such as Tn5 can be also used as a vector to introduce the object gene into the preferred host, especially on the host chromosome. DNA sequences containing any DNAs isolated from the preferred host together with the object gene are also useful to introduce the desired DNA sequence into the preferred host, especially on the host chromosome. Such DNA sequences can be transferred to the preferred host by transformation, transduction, transconjugation or electroporation.

[0078] Useful hosts are of prokaryotic or eukaryotic origin and may include organisms, mammalian cells, and plant cells. As a preferable organism, there may be mentioned bacteria such as *E. coli, P. putida, A. xylinum, A. pasteurianus, A. aceti, A. hansenii, G. oxydans*, and any Gram-negative bacteria which are capable of producing recombinant cytochrome c oxidase. Functional equivalents, subcultures, mutants and variants of said organism can be also used in the present invention. A preferred strain is *E. coli* K12 and its derivatives, *P. putida* or *G. oxydans* DSM 4025 and biologically or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of strain DSM 4025.

[0079] The DNA sequence encoding cytochrome c oxidase of the present invention is ligated into a suitable vector containing a regulatory region such as a promoter and a ribosomal binding site and transcriptional terminator operable in the host cell described above by well-known methods in the art to produce an expression vector.

[0080] To construct a host cell carrying an expression vector, various DNA transfer methods including transformation, transduction, conjugal mating (Chapters 14 and 15, Methods for General and Molecular Bacteriology, Philipp Gerhardt et al. ed., American Society for

Microbiology, 1994), and electroporation can be used. The method for constructing a transformed host cell may be selected from the methods well-known in the field of molecular biology. Usual transformation methods can be used for *E. coli*, *Pseudomonas* and *Acetobacter*. Transduction methods can also be used for *E. coli*. Conjugal mating system can be used in Gram-positive and Gram-negative bacteria including *E. coli*, *P. putida* and *G. oxydans*. A preferred conjugal mating method was disclosed in WO 89/06688. The conjugation can occur in liquid medium or on a solid surface. The preferred recipient for cytochrome c oxidase production is selected from *E. coli, P. putida* and *G. oxydans*. The preferred recipient for 2KGA production is selected from *E. coli, P. putida* and *G. oxydans*, which can produce active AADHs and cytochrome c with a suitable recombinant expression vector. The preferred recipient for 2KGA production is *G. oxydans* DSM 4025. A selective marker is usually added to the recipient in conjugal mating, for example, resistance against nalidixic acid or rifampicin is usually selected.

[0081] The following examples are provided to further illustrate the process of the present invention. These examples are illustrative only and are not intended to limit the scope of the invention in any way.

EXAMPLES

Example 1

Identification and purification of cytochrome c oxidase from *G. oxydans* DSM 4025

[0082] Terminal oxidase activity was spectrophotometrically measured using TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride) as an artificial substrate (electron donor). The reaction mixture consisted of 2.5 mM TMPD, 0.05% Tween-20 and 0.1 M sodium 3[N-morpholino]propanesulfonic acid (Na-MOPS) (pH 6.5). TMPD oxidase activity was measured as an increase in absorption at 520 nm, and the molar coefficient of TMPD was taken as 6.1 /mM/cm. One unit of enzyme activity was defined as 1 micromole oxidation of TMPD per one minute at room temperature. Spectrophotometric identification and quantification of a-type heme were carried out by analyzing a reduced minus oxidized difference spectrum to detect the characteristic positive peak around 605 nm. Each sample was reduced with sodium dithionite and oxidized with ammonium persulfate. The molar coefficient of the a-type heme peak (605 nm - 630 nm) was taken as 11.7 /mM/cm.

[0083] *G. oxydans* DSM 4025 was aerobically cultivated in 5 liters of FYC medium, consisting of 10% L-sorbose (sterilized separately), 0.05% glycerol, 1.6% urea (sterilized separately, 0.25% $MgSO_4x7H_2O$, 6.25% baker's yeast cells, 1.5% $CaCO_3$ (production grade, nacalai tesque, Kyoto, Japan), and 3.0% corn steep liquor, pH 7.5 (before sterilization), at 30°C for 27 hours. After the cultivation, solid materials such as $CaCO_3$ and yeast cells were precipitated by low speed centrifugation (1,000 rpm for 5 minutes) and removed. *G. oxydans* DSM 4025 cells remaining in the culture supernatant were collected by centrifugation at 8,000 rpm for 20 minutes and washed once with 25 mM sodium N-[2-hydroxyethyl] piperazine-N'-[4-butanesulfonic acid] (Na-HEPES) (pH 7.5) containing 0.25 M NaCl, and 2 mM $MgCl_2$.

[0084] The resulting cells (about 35 g wet weight) were suspended in about 200 ml of 25 mM Na-HEPES (pH 7.5) containing 0.5 mM ethylenediamine tetraacetic acid (EDTA), 0.5 mM ethylene glycol-bis-beta-aminoethyl ether (EGTA), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 microgram/ml pepstatin A, 1 microgram/ml leupeptin, 10 microgram/ml DNase I and 10 microgram/ml RNase A. The cell suspension was treated with a French press homogenizer at 1500 kg/cm^2 twice. The resulting suspension was centrifuged at 10,000 rpm for 10 minutes to remove cell debris, and the supernatant was collected as a cell-free extract (424.0 mg proteins). The cell-free extract was subjected to ultra-centrifugation at 55000 rpm for 1 hour to recover the precipitate as a crude membrane fraction. The crude membrane fraction was resuspended in 50 ml of 25 mM Na-HEPES (pH 7.5) containing 1.2% Tween 20, 0.25 M NaCl, 2 mM $MgCl_2$, 0.5 mM PMSF, 1 microgram/ml pepstatin A, 1 microgram/ml leupeptin and incubated for 1 hour to wash the membrane fraction. The fraction was again subjected to ultra-centrifugation at 55,000 rpm for 1 hour to recover the precipitate as a washed membrane fraction. The washed membrane fraction was incubated with 50 ml of 25 mM Na-HEPES (pH 7.5) containing 1.5% sucrose monolaulate (DOJIN Laboratories, Kumamoto, Japan), 2 mM EDTA and 5% glycerol for 1 hour to solubilize the membrane-bound proteins. The resulting suspension was subjected to ultra-centrifugation at 55,000 rpm for 1 hour to obtain a supernatant (50 ml) as a solubilized membrane fraction. Reduced minus oxidized difference spectrum of the solubilized membrane fraction displayed a characteristic positive peak around 605 nm; the peak corresponded to 0.41 nmoles of a-type heme/mg of crude proteins as content. The membrane-bound proteins in the solubilized membrane fraction were loaded on a DEAE-Toyopearl 650M (TOSOH, Tokyo, Japan) column (ID 2.2 x 5 cm) which had been equilibrated with 25 mM Na-HEPES containing 0.5% sucrose monolaurate and 5% glycerol. Fractionation was carried out by a linear gradient of 0 - 0.35 M NaCl in the same buffer. Fractions displaying an a-type heme spectra (positive peak around 605 nm on reduced

minus oxidized difference spectrum) and TMPD oxidase activity were eluted at around a 0.28 M concentration of NaCl. These fractions were collected (64 ml), dialyzed against 45 mM potassium phosphate buffer (KPB) (pH 7.6) containing 45 mM NaCl, 5% glycerol and 0.5% sucrose monolaurate, and the enzyme solution was loaded on a hydroxylapatite (TONEN Co., Tokyo, Japan) column (ID 1.5 x 6 cm) that had been equilibrated with the same buffer. The column was first washed with the same buffer and then with 500 mM KPB (pH 7.6) containing 500 mM NaCl, 5% glycerol and 0.5% sucrose monolaurate. The active fractions were eluted with 900 mM KPB (pH 7.6) containing 900 mM NaCl, 5% glycerol and 0.5% sucrose monolaurate, and collected. The fraction (8.6 mg protein) from the hydroxylapatite column was dialyzed against 25 mM Na-HEPES (pH 7.5) containing 0.5% sucrose monolaurate and 5% glycerol, concentrated by ultrafiltration using YM-30 membrane (Amicon Inc., MA, USA) and stored at –30°C as a purified protein.

[0085] The purified protein was subjected to native-polyacrylamide gel electrophoresis (Native-PAGE) analysis in the presence of 0.5% sucrose monolaurate. The purified protein displayed a visible band with greenish color (without protein staining) which corresponded to a single protein band (with protein staining). The purified protein had 2.6 units/mg of TMPD oxidase activity and displayed the typical absorption spectra pattern of aa3-type cytochrome c oxidase (Fig. 1). The concentration of a-type heme was estimated to be 19.2 nmoles/mg of purified protein. Purification of a-type heme from the washed membrane yielded a 100-fold increase in concentration with nearly 90% recovery. These results indicated that the purified protein was a major component exhibiting TMPD-oxidase activity in *G. oxydans* DSM 4025, and can function as a terminal oxidase in the respiratory system. SDS-PAGE analysis disassociated the purified protein into two protein components: one displayed a broad band with an apparent molecular weight of about 43,000 (named as CO I) and the other displayed a sharp band with an apparent molecular weight of about 36,000 (named as CO II); (Fig. 2).

Example 2

Amino acid sequence of the cytochrome c oxidase of *G. oxydans* DSM 4025 and the homologies with the other cytochrome c oxidase complexes

[0086] Two components (CO I and CO II) of the purified cytochrome c oxidase of *G. oxydans* DSM 4025 were disassociated by preparative SDS-PAGE. Native N-terminal amino acid sequences were not obtained from either component. To obtain the internal amino acid sequence, each component was digested with lysyl-endopeptidase and the resulting fragments were isolated by preparative SDS-PAGE and subjected to amino acid sequencing with an

amino acid sequencer (Applied Biosystems model 470A, The Perkin Elmer Corp., Conn., USA). Consequently, partial amino acid sequences were obtained; KDIGLLYLVAAGVVGF [SEQ ID NO: 11], was obtained from the CO I fragment (slightly lower molecular weight than the original) and KASQFTHNTPLEIVWTIVPV [SEQ ID NO:14], from the CO II fragment (about a 10000 lower molecular weight than the original). The partial amino acid sequences of the CO I and CO II subunits were compared with the total amino acid sequence of cytochrome c oxidase complexes of *P. denitrificans* and *R. sphaeroides* and bovine mitochondria by sequence-alignments (Figs. 3 to 4). These strains were chosen because CO I and CO II were similar to those of the cytochrome c oxidase of *P. dentrificans* (B. Ludwig and G. Schatz, Proc. Natl. Acad. Sci. USA, 77, 196-200, 1980) with regards to both their SDS-PAGE and spectrophotometric characteristics. Total homology in the amino acid sequences among three cytochrome c oxidase complexes had been previously reported (C. Jianli et al., J. Biol. Chem., 267, 24273-24278, 1992). As shown in Figs. 3 and 4, the amino acid sequences of *G. oxydans* DSM 4025 cytochrome c oxidase CO I and CO II were partially assigned to the homology alignment of the others. Especially, significant homology was observed with two bacterial sequences (*P. denitrificans* and *R. sphaeroides*).

Example 3

Cloning of cytochrome c oxidase genes of *G. oxydans* DSM 4025

(1) Amplification of partial cytochrome c oxidase gene(s) by the PCR method.

[0087] According to the total amino acid sequence alignments of *P. denitrificans*, *R. sphaeroides* and bovine mitochondria together with the amino acid sequences of the purified CO I and CO II polypeptides (SEQ ID NOs: 11 and 14), the following amino acid sequences were selected for PCR primers to amplify partial DNA sequences of CO I and CO II genes: SEQ ID NO: 9 and SEQ ID NO:10 for the CO I gene; and SEQ ID NO: 15 and SEQ ID NO:16 for the CO II gene. The third component (CO III), which was reported to be included in THE cytochrome c oxidase complex, did not exist in the preparation purified from *G. oxydans* DSM 4025. the absence OF CO III seemed to be due to the disassociation of the complex during purification. To confirm and amplify a partial DNA sequence encoding the assumed CO III gene of *G. oxydans* DSM 4025, if it in fact exists, two amino acid sequences corresponding to the conserved regions of the polypeptides encoded by the CO III genes of *P. denitrificans*, *R. sphaeroides* and bovine mitochondria, were selected (Fig. 5.) : SEQ ID NO: 17 and SEQ ID NO:18 for the CO III gene. Each pair of primers was specifically designed for CO I, CO II or

CO III (Fig. 6). The PCR reaction was carried out by using the GeneAmp™ DNA Amplification Reagent Kit (Takara Shuzo, Kyoto, Japan) with the Perkin-Elmer Cetus Instruments Thermal Cycler according to the recommendations of the supplier. The reaction consisted of 30 cycles of 1) a denaturation step at 94°C for 1 minute; 2) an annealing step at 42 or 50°C for 2 minutes; and 3) a synthesis step at 72°C for 3 minutes. The reaction mixture (100 microliter) contained 200 micromole of dNTPs, 2.9 micromole (for 32 degeneracy) or 5.8 micromole (for 64 degeneracy) of each primer, 2.2 ng of chromosomal DNA of *G. oxydans* DSM 4025, and 2.5 units of Taq polymerase in the buffer supplied. PCR product was detected by agarose gel electrophoresis (AGE) with ethidium bromide staining. As a result, DNA fragments of expected length (about 180 bp for CO I, about 180 bp for CO II, about 300 bp for CO III) were amplified.

(2) Cloning and nucleotide sequencing of the DNA fragments amplified by PCR.

[0088] The PCR-amplified DNA fragments were purified from an agarose gel and directly cloned into the pCRTMII vector (Invitrogen Corporation, USA), and the DNA sequences were determined according to the supplier's instruction. Amino acid sequences deduced from the nucleotide sequences of the PCR products displayed considerable homology with the sequences of target positions in the sequence alignments (Figs. 3 to 5). The PCR products encoding the partial amino acid sequences of CO I, CO II and CO III were labeled with ^{32}P to obtain probes Pco1, Pco2, and Pco3, respectively. The probes were used for Southern- or colony-hybridization to detect the complete CO I, CO II, and CO III genes.

(3) Southern-blot analysis of the *G. oxydans* DSM 4025 chromosomal DNA using the PCR products as probes.

[0089] The chromosomal DNA of *G. oxydans* DSM 4025 digested with various restriction endonucleases was subjected to Southern hybridization using the probes. The probe Pco1 hybridized to a Pst I fragment (8.0 kb), and the probes Pco2 and Pco3 hybridized to an EcoRI fragment (9.3 kb), of the chromosomal DNA.

(4) Cloning of complete cytochrome c oxidase genes in the 8.0 kb PstI fragment (CO I) and the 9.3 kb EcoRI fragment (CO II and CO III).

[0090] The chromosomal DNA of *G. oxydans* DSM 4025 was completely digested with PstI or EcoRI and the resulting fragments were subjected to agarose gel electrophoresis. EcoRI-digests around 9.3 kb (7-12 kb) in size and PstI-digests around 8 kb (6-10 kb) were cut

out and eluted from the gel. The recovered DNA fragments were ligated with PstI- or EcoRI-digested pUC19 vector to transform *E. coli* JM109. About 1,000 transformants were obtained as a PstI- or EcoRI-library. Colony hybridization was performed with the probe Pco1 on the PstI library and with the primers Pco2 and Pco3 on the EcoRI library. From each library, several positive colonies were obtained. Plasmid DNAs were extracted from the colonies and digested with PstI or EcoRI; an 8.0 kb PstI fragment showed a strong signal with the probe Pco1, and a 9.3 kb EcoRI fragment showed a strong signal with both of the probes Pco2 and Pco3. The plasmid containing 8.0 kb PstI fragment was designated as pUCO01 and the plasmid containing the 9.3 kb EcoRI fragment as pUCO23.

(5) Physical map of the 8.0 kb PstI and 9.3 kb EcoRI fragments.

[0091] Physical maps of the 8.0 kb PstI and 9.3 kb EcoRI fragments were constructed by Southern hybridization analysis of the fragments digested with various restriction endonucleases with the probes Pco1, Pco2 adn Pco3. Direction and distance of the CO II and CO III genes encoded on the 9.3 kb EcoRI fragment were determined by the PCR method with primers derived from the partial nucleotide sequences (Fig. 7).

(6) Nucleotide sequencing of the complete CO I gene.

[0092] The nucleotide sequence of the COI gene on pUCO01 was determined by the dideoxy chain termination method. A 2.9 kb fragment upstream from a HindIII site, as shown in Fig. 7, was sequenced and one open reading frame (CDS of 1,674 bp existing in the sequence shown in SEQ ID NO: 1) was found in the fragment. This ORF encodes a protein of 558 amino acids (sequence list SEQ ID NO: 2), containing the amino acid stretch consistent with the amino acid sequence (SEQ ID NO: 11) of the peptide fragment derived from the purified CO I and the amino acid sequence (SEQ ID NO: 13) deduced from the DNA sequence of the about-180 bp PCR product (SEQ ID NO: 12) for CO I (see 3-(1)). The CO I amino acid sequence of *G. oxydans* DSM 4025 displayed 78.7, 76.0 and 53.3% homology with those of *R. sphaeroides, P. denitrificans* and bovine mitochondria, respectively. (Fig. 8)

(7) Construction of an expression plasmid encoding all of the CO I, CO II, and CO III genes.

[0093] The CO I gene was isolated from the 8.0 kb PstI fragment on pUCO01 by complete HindIII- and partial-EcoRI digestion, as a 3.5 kb fragment (Fig. 7). According to the physical map of the 9.3 kb EcoRI fragment on pUCO23, the CO II and CO III genes were isolated by complete-KpnI and partial-PstI digestions yielding a 6.0 kb fragment in tandem form (Fig. 7).

Each fragment was independently subcloned into the BluescriptII SK+ vector to obtain plasmids pBCO01 with a 3.5 kb fragment containing the CO I gene, and pBCO23 with a 6.0 kb fragment containing the CO II and CO III genes.

[0094] As shown in Fig. 9, the 3.5 kb fragment containing the CO I gene and the 6.0 kb fragment containing the CO II and CO III genes were co-integrated in one expression vector for the functional expression of the genes of the cytochrome c oxidase complex (CO I, CO II, and CO III). First, the 6.0 kb XbaI - KpnI fragment the containing CO II and CO III genes from pBCO23 was inserted in the EcoRI site of the plasmid vector, pVK101 by blunt end ligation. Then, the 3.5 kb XbaI - HindIII fragment containing the CO I gene from pBCO01 was inserted in the BglII site of the pVK101 vector that already contained the 6.0 kb XbaI - KpnI fragment. The resulting plasmid vector was designated as pVKcoxes.

Example 4

Overexpression of cytochrome c oxidase genes in a IDSM 4025 derivative

[0095] The plasmid carrying the cytochrome c oxidase genes in pVK101, pVKcoxes, was introduced into a rifampicin resistant derivative of *G. oxydans* DSM 4025, GOS2RPM (a single colony isolate from GOS2R; T. Hoshino et al., European Patent Publication 832974 A2) by the tri-parental conjugal mating method. Cells of GOS2RPM were cultivated at 30°C in 10 ml of T medium consisting of 3% Trypticase Soy Broth (Becton Dickinson, Cockeysville, Md., USA) and 0.3% yeast extract (Difco Laboratories, Detroit, Mich.) with 100 microgram/ml of rifampicin (TR medium). A donor strain, *E. coli* HB carrying pVKcoxes (Tc^r, Km^r) or pVK102 (Tc^r, Km^r) and a helper strain, *E. coli* HB101 carrying pRK2013 (Km^r) were grown in *Luria Bertani* medium containing appropriate antibiotics overnight at 37°C. These overnight cultures (10 ml of GOS2RPM culture and 2 ml of *E. coli* culture) were independently centrifuged and cell pellets were independently suspended in 2 ml of T medium. One hundred microliter of the cell suspensions were mixed and 50 microliter of the mixed cell suspension was spotted onto a nitrocellulose filter placed on the surface of NS2 agar medium consisting of 5.0% D-mannitol, 0.25% $MgSO_4 \cdot 7H_2O$, 1.75% corn steep liquor, 5% baker's yeast (Oriental Yeast Co., Tokyo, Japan), 0.5% $CaCO_3$, 0.5% urea (separately sterilized), and 2.0% agar, pH 7.0 (before sterilization). The plate was incubated at 27°C overnight. The resulting cells were spread onto T agar medium containing 100 microgram/ml rifampicin and 3 microgram/ml tetracycline (TRT agar plate). The transconjugants thus obtained were purified by streaking on TRT agar plates to remove cells of *E. coli* and plasmid-free GOS2RPM.

[0096] The resulting transconjugants, GOS2RPM (pVKcoxes) and GOS2R (pVK102) were cultivated, and cells of both transconjugants were prepared according to the method described in Example 1. The cytochrome c oxidase levels in GOS2RPM (pVKcoxes) and GOS2RPM (pVK102) were determined by the following experiments and compared with each other. From both strains, solubilized membrane fractions were prepared by the method described in Example 1. First, a-type heme contents were determined to be 0.031 and 0.022 nmoles/mg of cell proteins for GOS2RPM (pVKcoxes) and GOS2R (pVK102), respectively, by the reduced minus oxidized difference spectrum method (Example 1). Second, the specific oxidation rate of cytochrome c (purchased from Sigma, horse heart type VI) was measured. Reduced cytochrome c was prepared by using sodium dithinite as a reducer, with excess reducer removed by two treatments with a PD-10 column (Pharmacia). The reaction mixture consisted of 33 mM reduced cytochrome c, 25 mM Na-HEPES (pH 7.2), 2% sucrose monolaurate and 0.5 mM EDTA. The oxidation rate of the reduced cytochrome c was measured as a decrease in absorbance at 550 nm with the molar coefficient taken as 21.1 /mM/cm. Specific oxidation rates on the reduced cytochrome c were determined to be 1.58 and 2.00 nmoles/mg cell proteins/min for GOS2RPM (pVK102) and GOS2R (pVKcoxes), respectively. Third, the amounts of CO I and CO II components were compared by Western-blot analysis with antibodies against the CO I or CO II components. Stronger band intensities (60% increase for CO I and 41% increase for CO II as measured with a CCD camera) were observed on GOS2RPM (pVKcoxes). These results suggested that introduction of pVKcoxes resulted in functional amplification of the cytochrome c oxidase complex level in the 2KGA producing *G. oxydans* DSM 4025 derivative.

[0097] The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention and all such modifications are intended to be included within the scope of the following claims.